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(54) Title: POLYUNSATURATED FATTY ACIDS IN PLANTS

(57) Abstract

The present invention relates to compositions and methods for preparing polyunsaturated long chain fatty acids in plants, plant parts and plant cells, such as leaves, roots, fruits and seeds. Nucleic acid sequences and constructs encoding fatty acid desaturases, including Δ5–desaturases, Δ6–desaturases and Δ12–desaturases, are used to generate transgenic plants, plant parts and cells which contain and express one or more transgenes encoding one or more desaturases. Expression of the desaturases with different substrate specificities in the plant system permits the large scale production of polyunsaturated long chain fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, α–linolenic acid, gamma–linolenic acid, arachidonic acid and the like for modification of the fatty acid profile of plants, plant parts and tissues. Manipulation of the fatty acid profiles allows for the production of commercial quantities of novel plant oils and products.

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POLYUNSATURATED FATTY ACIDS IN PLANTS

Field of the Invention

This invention relates to modulating levels of enzymes and/or enzyme components capable of altering the production of long chain polyunsaturated fatty acids (PUFAS) in a host plant. The invention is exemplified by the production of PUFAS in plants.

Background

Three main families of polyunsaturated fatty acids (PUFAs) are the 3 fatty acids, exemplified by arachidonic acid, the ω9 fatty acids exemplified by Mead acid, and the ω6 fatty acids, exemplified by eicosapentaenoic acid. PUFAs are important components of the plasma membrane of the cell, where they may be found in such forms as phospholipids. PUFAs also serve as precursors to other molecules of importance in human beings and animals, including the prostacyclins, leukotrienes and prostaglandins. PUFAs are necessary for proper development, particularly in the developing infant brain, and for tissue formation and repair.

Four major long chain PUFAs of importance include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are primarily found in different types of fish oil, gamma-linolenic acid (GLA), which is found in the seeds of a number of plants, including evening primrose (*Oenothera biennis*), borage (*Borago officinalis*) and black currants (*Ribes nigrum*), and stearidonic acid (SDA), which is found in marine oils and plant seeds. Both GLA and another important long chain PUFA, arachidonic acid (ARA), are found in filamentous fungi. ARA can be purified from animal tissues including liver and adrenal gland. Mead acid accumulates in essential fatty acid deficient animals.

For DHA, a number of sources exist for commercial production including a variety of marine organisms, oils obtained from cold water marine fish, and egg yolk fractions. For ARA, microorganisms including the genera *Mortierella, Entomophthora, Phytium* and *Porphyridium* can be used for commercial production. Commercial sources of SDA include the genera *Trichodesma and*

Echium. Commercial sources of GLA include evening primrose, black currants and borage. However, there are several disadvantages associated with commercial production of PUFAs from natural sources. Natural sources of PUFAs, such as animals and plants, tend to have highly heterogeneous oil compositions. The oils obtained from these sources therefore can require extensive purification to separate out one or more desired PUFAs or to produce an oil which is enriched in one or more PUFA. Natural sources also are subject to uncontrollable fluctuations in availability. Fish stocks may undergo natural variation or may be depleted by overfishing. Fish oils have unpleasant tastes and odors, which may be impossible to economically separate from the desired product, and can render such products unacceptable as food supplements. Animal oils, and particularly fish oils, can accumulate environmental pollutants. Weather and disease can cause fluctuation in yields from both fish and plant sources. Cropland available for production of alternate oil-producing crops is subject to competition from the steady expansion of human populations and the associated increased need for food production on the remaining arable land. Crops which do produce PUFAs, such as borage, have not been adapted to commercial growth and may not perform well in monoculture. Growth of such crops is thus not economically competitive where more profitable and better established crops can be grown. Large scale fermentation of organisms such as *Mortierella* is also expensive. Natural animal tissues contain low amounts of ARA and are difficult to process. Microorganisms such as *Porphyridium* and *Mortierella* are difficult to cultivate on a commercial scale.

Dietary supplements and pharmaceutical formulations containing PUFAs can retain the disadvantages of the PUFA source. Supplements such as fish oil capsules can contain low levels of the particular desired component and thus require large dosages. High dosages result in ingestion of high levels of undesired components, including contaminants. Care must be taken in providing fatty acid supplements, as overaddition may result in suppression of endogenous biosynthetic pathways and lead to competition with other necessary fatty acids in various lipid fractions *in vivo*, leading to undesirable results. For example, Eskimos having a diet high in ω3 fatty acids have an increased tendency to bleed (U.S. Pat. No.

4,874,603). Unpleasant tastes and odors of the supplements can make such regimens undesirable, and may inhibit compliance by the patient.

A number of enzymes are involved in PUFA biosynthesis. Linoleic acid (LA, 18:2 Δ9, 12) is produced from oleic acid (18:1 Δ9) by a Δ12-desaturase. GLA (18:3 Δ6, 9, 12) is produced from linoleic acid (LA, 18:2 Δ9, 12) by a Δ6-desaturase. ARA (20:4 Δ5, 8, 11, 14) production from DGLA (20:3 Δ8, 11, 14) is catalyzed by a Δ5-desaturase. However, animals cannot desaturate beyond the Δ9 position and therefore cannot convert oleic acid (18:1 Δ9) into linoleic acid (18:2 Δ9, 12). Likewise, α-linolenic acid (ALA, 18:3 Δ9, 12, 15) cannot be synthesized by mammals. Other eukaryotes, including fungi and plants, have enzymes which desaturate at positions Δ21 and Δ15. The major poly-unsaturated fatty acids of animals therefore are either derived from diet and/or from desaturation and elongation of linoleic acid (18:2 Δ9, 12) or α-linolenic acid (18:3 Δ9, 12, 15).

Poly-unsaturated fatty acids are considered to be useful for nutritional, pharmaceutical, industrial, and other purposes. An expansive supply of poly-unsaturated fatty acids from natural sources and from chemical synthesis are not sufficient for commercial needs. Therefore it is of interest to obtain genetic material involved in PUFA biosynthesis from species that naturally produce these fatty acids and to express the isolated material alone or in combination in a heterologous system which can be manipulated to allow production of commercial quantities of PUFAS.

SUMMARY OF THE INVENTION

Novel compositions and methods are provided for preparation of poly-unsaturated long chain fatty acids and desaturases in plants and plant cells. The methods involve growing a host plant cell of interest transformed with an expression cassette functional in a host plant cell, the expression cassette comprising a transcriptional and translational initiation regulatory region, joined in reading frame 5' to a DNA sequence encoding a desaturase polypeptide capable of modulating the production of PUFAs. Expression of the desaturase polypeptide provides for an alteration in the PUFA profile of host plant cells as a result of

altered concentrations of enzymes involved in PUFA biosynthesis. Of particular interest is the selective control of PUFA production in plant tissues and/or plant parts such as leaves, roots, fruits and seeds. The invention finds use for example in the large scale production of DHA, Mead Acid EPA, ARA, Stearidonic acid and GLA and for modification of the fatty acid profile of edible plant tissues and/or plant parts.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows possible pathways for the synthesis of Mead acid (20:3 Δ5, 8, 11), arachidonic acid (20:4 Δ5, 8, 11, 14) and stearidonic acid (18:4 Δ6, 9, 12, 15) from palmitic acid (C_{16}) from a variety of organisms, including algae, *Mortierella* and humans. These PUFAs can serve as precursors to other molecules important for humans and other animals, including prostacyclins, leukotrienes, and prostaglandins, some of which are shown.

Figure 2 shows possible pathways for production of PUFAs in addition to ARA, including taxoleic acid and pinolenic, again compiled from a variety of organisms.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

SEQ ID NO:1 shows DNA sequence from a *Schizochytrium* clone with homology to both Δ12 and Δ15 desaturases.

SEQ ID NO 2 shows peptide sequence from a *Schizochytrium* clone with homology to both Δ12 and Δ15 desaturases.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In order to ensure a complete understanding of the invention, the following definitions are provided:

Δ5-Desaturase: Δ5 desaturase is an enzyme which introduces a double bond between carbons 5 and 6 from the carboxyl end of a fatty acid molecule.

Δ6-Desaturase: Δ6-desaturase is an enzyme which introduces a double bond between carbons 6 and 7 from the carboxyl end of a fatty acid molecule.

Δ9-Desaturase: Δ9-desaturase is an enzyme which introduces a double bond between carbons 9 and 10 from the carboxyl end of a fatty acid molecule.

Δ12-Desaturase: Δ12-desaturase is an enzyme which introduces a double bond between carbons 12 and 13 from the carboxyl end of a fatty acid molecule.

Fatty Acids: Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. Fatty acids include the following:

Fatty Acid		
12:0	lauric acid	
16:0	Palmitic acid	
16:1	Palmitoleic acid	
18:0	stearic acid	
18:1	oleic acid	Δ9-18:1
18:2 Δ5,9	Taxoleic acid	Δ5,9-18:2
18:2 Δ6,9	6,9-octadecadienoic acid	Δ6,9-18:2
18:2	Linoleic acid	Δ9,12-18:2 (LA)
18:3 Δ6,9,12	Gamma-linolenic acid	Δ6,9,12-18:3 (GLA)
18:3 Δ5,9,12	Pinolenic acid	Δ5,9,12-18:3
18:3	alpha-linolenic acid	Δ9,12,15-18:3 (ALA)
18:4	Stearidonic acid	Δ6,9,12,15-18:4 (SDA)
20:0	Arachidic acid	
20:1	Eicosenic Acid	
20:2 Δ8, 11		Δ8, 11
20:3 Δ5, 8, 11	Mead Acid	Δ5, 8, 11
22:0	Behehic acid	
22:1	erucic acid	
22:2	Docasadienoic acid	
20:4 ω6	arachidonic acid	Δ5,8,11,14-20:4 (ARA)
20:3 ω6	ω6-eicosatrienoic dihomo-gamma linolenic	Δ8,11,14-20:3 (DGLA)
20:5 ω3	Eicosapentanoic (Timnodonic acid)	Δ5,8,11,14,17-20:5 (EPA)
20:3 ω3	ω3-eicosatrienoic	Δ11,16,17-20:3
20:4 ω3	ω3-eicosatetraenoic	Δ8,11,14,17-20:4
22:5 ω3	Docosapentaenoic	Δ7,10,13,16,19-22:5 (ω3DPA)

Fatty Acid		
22:6 ω3	Docosahexaenoic (cervonic acid)	Δ4,7,10,13,16,19-22:6 (DHA)
24:0	Lignoceric acid	

Taking into account these definitions, the present invention is directed to novel DNA sequences, DNA constructs, methods and compositions are provided which permit modification of the poly-unsaturated long chain fatty acid content of plant cells. Plant cells are transformed with an expression cassette comprising a DNA encoding a polypeptide capable of increasing the amount of one or more PUFA in a plant cell. Desirably, integration constructs may be prepared which provide for integration of the expression cassette into the genome of a host cell. Host cells are manipulated to express a sense or antisense DNA encoding a polypeptide(s) that has desaturase activity. By "desaturase" is intended a polypeptide which can desaturate one or more fatty acids to produce a mono- or poly-unsaturated fatty acid or precursor thereof of interest. By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification, for example, glycosylation or phosphorylation. The substrate(s) for the expressed enzyme may be produced by the host cell or may be exogenously supplied.

To achieve expression in a host cell, the transformed DNA is operably associated with transcriptional and translational initiation and termination regulatory regions that are functional in the host cell. Constructs comprising the gene to be expressed can provide for integration into the genome of the host cell or can autonomously replicate in the host cell. For production of linoleic acid (LA), the expression cassettes generally used include a cassette which provides for Δ12 desaturase activity, particularly in a host cell which produces or can take up oleic acid. For production of ALA, the expression cassettes generally used include a cassette which provides for Δ15 or ω3 desaturase activity, particularly in a host cell which produces or can take up LA. For production of GLA or SDA, the expression cassettes generally used include a cassette which provides for Δ6 desaturase activity, particularly in a host cell which produces or can take up LA or ALA.

respectively. Production of ω6-type unsaturated fatty acids, such as LA or GLA, is favored in a plant capable of producing ALA by inhibiting the activity of a Δ15 or ω3 type desaturase; this is accomplished by providing an expression cassette for an antisense Δ15 or ω3 transcript, or by disrupting a Δ15 or ω3 desaturase gene. Similarly, production of LA or ALA is favored in a plant having Δ6 desaturase activity by providing an expression cassette for an antisense Δ6 transcript, or by disrupting a Δ6 desaturase gene. Production of oleic acid likewise is favored in a plant having Δ12 desaturase activity by providing an expression cassette for an antisense Δ12 transcript, or by disrupting a Δ12 desaturase gene. For production of ARA, the expression cassette generally used provides for Δ5 desaturase activity, particularly in a host cell which produces or can take up DGLA. Production of ω6-type unsaturated fatty acids, such as ARA, is favored in a plant capable of producing ALA by inhibiting the activity of a Δ15 or ω3 type desaturase; this is accomplished by providing an expression cassette for an antisense Δ15 or ω3 transcript, or by disrupting a Δ15 or ω3 desaturase gene.

TRANSGENIC PLANT PRODUCTION OF FATTY ACIDS

Transgenic plant production of PUFAs offers several advantages over purification from natural sources such as fish or plants. Production of fatty acids from recombinant plants provides the ability to alter the naturally occurring plant fatty acid profile by providing new synthetic pathways in the host or by suppressing undesired pathways, thereby increasing levels of desired PUFAs, or conjugated forms thereof, and decreasing levels of undesired PUFAs. Production of fatty acids in transgenic plants also offers the advantage that expression of desaturase genes in particular tissues and/or plant parts means that greatly increased levels of desired PUFAs in those tissues and/or parts can be achieved, making recovery from those tissues more economical. For example, the desired PUFAs can be expressed in seed; methods of isolating seed oils are well established. In addition to providing a source for purification of desired PUFAs, seed oil components can be manipulated through expression of desaturase genes, either alone or in combination with other genes such as elongases, to provide seed oils having a particular PUFA profile in concentrated form. The concentrated seed oils then can be added to animal milks

and/or synthetic or semi-synthetic milks to serve as infant formulas where human nursing is impossible or undesired, or in cases of malnourishment or disease in both adults and infants.

For production of PUFAs, depending upon the host cell, the availability of substrate, and the desired end product(s), several polypeptides, particularly desaturases, are of interest including those polypeptides which catalyze the conversion of stearic acid to oleic acid, LA to GLA, of ALA to SDA, of oleic acid to LA, or of LA to ALA, which includes enzymes which desaturate at the Δ6, Δ9, Δ12, Δ15 or ω3 positions. Considerations for choosing a specific polypeptide having desaturase activity include the pH optimum of the polypeptide, whether the polypeptide is a rate limiting enzyme or a component thereof, whether the desaturase used is essential for synthesis of a desired poly-unsaturated fatty acid, and/or co-factors required by the polypeptide. The expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell. For example, the polypeptide may have to compete for substrate with other enzymes in the host cell. Analyses of the K_m and specific activity of the polypeptide in question therefore are considered in determining the suitability of a given polypeptide for modifying PUFA production in a given host cell. The polypeptide used in a particular situation therefore is one which can function under the conditions present in the intended host cell but otherwise can be any polypeptide having desaturase activity which has the desired characteristic of being capable of modifying the relative production of a desired PUFA. A scheme for the synthesis of arachidonic acid (20:4 Δ5, 8, 11, 14) from palmitic acid (C_{16}) is shown in Figure 1. A key enzyme in this pathway is a Δ5-desaturase which converts DH-γ-linolenic acid (DGLA, eicosatrienoic acid) to ARA. Conversion of α-linolenic acid (ALA) to stearidonic acid by a Δ6-desaturase is also shown. Production of PUFAs in addition to ARA, including EPA and DHA is shown in Figure 2. A key enzyme in the synthesis of arachidonic acid (20:4 Δ5, 8, 11, 14) from stearic acid (C_{18}) is a Δ6-desaturase which converts the linoleic acid into γ-linolenic acid. Conversion of α-linolenic acid (ALA) to stearidonic acid by a Δ6-desaturase also is shown. For production of ARA, the DNA sequence used encodes

a polypeptide having Δ5 desaturase activity. In particular instances, this can be coupled with an expression cassette which provides for production of a polypeptide having Δ6 desaturase activity and, optionally, a transcription cassette providing for production of antisense sequences to a Δ 15 transcription product. The choice of combination of cassettes used depends in part on the PUFA profile of the host cell. Where the host cell Δ5-desaturase activity is limiting, overexpression of Δ5 desaturase alone generally will be sufficient to provide for enhanced ARA production.

SOURCES OF POLYPEPTIDES HAVING DESATURASE ACTIVITY

As sources of polypeptides having desaturase activity and oligonucleotides encoding such polypeptides are organisms which produce a desired poly-unsaturated fatty acid. As an example, microorganisms having an ability to produce ARA can be used as a source of Δ5-desaturase genes; microorganisms which GLA or SDA can be used as a source of Δ6-desaturase and/or Δ12-desaturase genes. Such microorganisms include, for example, those belonging to the genera *Mortierella, Conidiobolus, Pythium, Phytophathora, Penicillium, Porphyridium, Coidosporium, Mucor, Fusarium, Aspergillus, Rhodotorula,* and *Entomophthora*. Within the genus *Porphyridium*, of particular interest is *Porphyridium cruentum*. Within the genus *Mortierella*, of particular interest are *Mortierella elongata, Mortierella exigua, Mortierella hygrophila, Mortierella ramanniana*, var. angulispora, and *Mortierella alpina*. Within the genus *Mucor*, of particular interest are *Mucor circinelloides* and *Mucor javanicus*.

DNAs encoding desired desaturases can be identified in a variety of ways. As an example, a source of the desired desaturase, for example genomic or cDNA libraries from *Mortierella*, is screened with detectable enzymatically- or chemically-synthesized probes, which can be made from DNA, RNA, or non-naturally occurring nucleotides, or mixtures thereof. Probes may be enzymatically synthesized from DNAs of known desaturases for normal or reduced-stringency hybridization methods. Oligonucleotide probes also can be used to screen sources and can be based on sequences of known desaturases, including sequences

conserved among known desaturases, or on peptide sequences obtained from the desired purified protein. Oligonucleotide probes based on amino acid sequences can be degenerate to encompass the degeneracy of the genetic code, or can be biased in favor of the preferred codons of the source organism. Oligonucleotides also can be used as primers for PCR from reverse transcribed mRNA from a known or suspected source; the PCR product can be the full length cDNA or can be used to generate a probe to obtain the desired full length cDNA. Alternatively, a desired protein can be entirely sequenced and total synthesis of a DNA encoding that polypeptide performed.

Once the desired genomic or cDNA has been isolated, it can be sequenced by known methods. It is recognized in the art that such methods are subject to errors, such that multiple sequencing of the same region is routine and is still expected to lead to measurable rates of mistakes in the resulting deduced sequence, particularly in regions having repeated domains, extensive secondary structure, or unusual base compositions, such as regions with high GC base content. When discrepancies arise, resequencing can be done and can employ special methods. Special methods can include altering sequencing conditions by using: different temperatures; different enzymes; proteins which alter the ability of oligonucleotides to form higher order structures; altered nucleotides such as ITP or methylated dGTP; different gel compositions, for example adding formamide; different primers or primers located at different distances from the problem region; or different templates such as single stranded DNAs. Sequencing of mRNA can also be employed.

For the most part, some or all of the coding sequence for the polypeptide having desaturase activity is from a natural source. In some situations, however, it is desirable to modify all or a portion of the codons, for example, to enhance expression, by employing host preferred codons. Host preferred codons can be determined from the codons of highest frequency in the proteins expressed in the largest amount in a particular host species of interest. Thus, the coding sequence for a polypeptide having desaturase activity can be synthesized in whole or in part. All or portions of the DNA also can be synthesized to remove any destabilizing sequences or regions of secondary structure which would be present in the

transcribed mRNA. All or portions of the DNA also can be synthesized to alter the base composition to one more preferable in the desired host cell. Methods for synthesizing sequences and bringing sequences together are well established in the literature. *In vitro* mutagenesis and selection, site-directed mutagenesis, or other means can be employed to obtain mutations of naturally occurring desaturase genes to produce a polypeptide having desaturase activity *in vivo* with more desirable physical and kinetic parameters for function in the host cell, such as a longer half-life or a higher rate of production of a desired polyunsaturated fatty acid.

Desirable cDNAs have less than 60% A+T composition, preferably less than 50% A+T composition. On a localized scale of a sliding window of 20 base pairs, it is preferable that there are no localized regions of the cDNA with greater than 75% A+T composition; with a window of 60 base pairs, it is preferable that there are no localized regions of the cDNA with greater than 60%, more preferably no localized regions with greater than 55% A+T composition.

***Mortierella alpina* Desaturases**

Of particular interest are the *Mortierella alpina* Δ5-desaturase, Δ6-desaturase, Δ12-desaturase and Δ15 desaturase. The gene encoding the *Mortierella alpina* Δ5-desaturase can be expressed in transgenic plants to effect greater synthesis of ARA from DGLA. Other DNAs which are substantially identical in sequence to the *Mortierella alpina* Δ5-desaturase DNA, or which encode polypeptides which are substantially identical in sequence to the *Mortierella alpina* Δ5-desaturase polypeptide, also can be used. The gene encoding the *Mortierella alpina* Δ6-desaturase can be expressed in transgenic plants or animals to effect greater synthesis of GLA from linoleic acid or of stearidonic acid (SDA) from ALA. Other DNAs which are substantially identical in sequence to the *Mortierella alpina* Δ6-desaturase DNA, or which encode polypeptides which are substantially identical in sequence to the *Mortierella alpina* Δ6-desaturase polypeptide, also can be used.

The gene encoding the *Mortierella alpina* Δ12-desaturase can be expressed in transgenic plants to effect greater synthesis of LA from oleic acid. Other DNAs

which are substantially identical to the *Mortierella alpina* Δ12-desaturase DNA, or which encode polypeptides which are substantially identical to the *Mortierella alpina* Δ12-desaturase polypeptide, also can be used.

By substantially identical in sequence is intended an amino acid sequence or nucleic acid sequence exhibiting in order of increasing preference at least 60%, 80%, 90% or 95% homology to the *Mortierella alpina* Δ5-desaturase amino acid sequence or nucleic acid sequence encoding the amino acid sequence. For polypeptides, the length of comparison sequences generally is at least 16 amino acids, preferably at least 20 amino acids, or most preferably 35 amino acids. For nucleic acids, the length of comparison sequences generally is at least 50 nucleotides, preferably at least 60 nucleotides, and more preferably at least 75 nucleotides, and most preferably, 110 nucleotides. Homology typically is measured using sequence analysis software, for example, the Sequence Analysis software package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin 53705, MEGAlign (DNAStar, Inc., 1228 S. Park St., Madison, Wisconsin 53715), and MacVector (Oxford Molecular Group, 2105 S. Bascom Avenue, Suite 200, Campbell, California 95008). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. Substitutions may also be made on the basis of conserved hydrophobicity or hydrophilicity (Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982), or on the basis of the ability to assume similar polypeptide secondary structure (Chou and Fasman, *Adv. Enzymol.* 47: 45-148, 1978).

EXPRESSION OF DESATURASE GENES

Once the DNA encoding a desaturase polypeptide has been obtained, it is placed in a vector capable of replication in a host cell, or is propagated *in vitro* by means of techniques such as PCR or long PCR. Replicating vectors can include plasmids, phage, viruses, cosmids and the like. Desirable vectors include those

useful for mutagenesis of the gene of interest or for expression of the gene of interest in host cells. The technique of long PCR has made *in vitro* propagation of large constructs possible, so that modifications to the gene of interest, such as mutagenesis or addition of expression signals, and propagation of the resulting constructs can occur entirely *in vitro* without the use of a replicating vector or a host cell.

For expression of a desaturase polypeptide, functional transcriptional and translational initiation and termination regions are operably linked to the DNA encoding the desaturase polypeptide. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the desired system, expression vectors, chemical synthesis, or from an endogenous locus in a host cell. Expression in a plant tissue and/or plant part presents certain efficiencies, particularly where the tissue or part is one which is easily harvested, such as seed, leaves, fruits, flowers, roots, etc. Expression can be targeted to that location within the plant by using specific regulatory sequences, such as those of USPN 5,463,174, USPN 4,943,674, USPN 5,106,739, USPN 5,175,095, USPN 5,420,034, USPN 5,188,958, and USPN 5,589,379. Alternatively, the expressed protein can be an enzyme which produces a product which may be incorporated, either directly or upon further modifications, into a fluid fraction from the host plant. In the present case, expression of desaturase genes, or antisense desaturase transcripts, can alter the levels of specific PUFAs, or derivatives thereof, found in plant parts and/or plant tissues. The Δ5-desaturase polypeptide coding region is expressed either by itself or with other genes, in order to produce tissues and/or plant parts containing higher proportions of desired PUFAs or in which the PUFA composition more closely resembles that of human breast milk (Prieto *et al.*, PCT publication WO 95/24494). The termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known to and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region usually is selected more as a matter of convenience rather than because of any particular property.

The choice of a host cell is influenced in part by the desired PUFA profile of the transgenic cell, and the native profile of the host cell. As an example, for production of linoleic acid from oleic acid, the DNA sequence used encodes a polypeptide having Δ12 desaturase activity, and for production of GLA from linoleic acid, the DNA sequence used encodes a polypeptide having Δ6 desaturase activity. Use of a host cell which expresses Δ12 desaturase activity and lacks or is depleted in Δ15 desaturase activity, can be used with an expression cassette which provides for overexpression of Δ6 desaturase alone generally is sufficient to provide for enhanced GLA production in the transgenic cell. Where the host cell expresses Δ9 desaturase activity, expression of both a Δ12- and a Δ6-desaturase can provide for enhanced GLA production. In particular instances where expression of Δ6 desaturase activity is coupled with expression of Δ12 desaturase activity, it is desirable that the host cell naturally have, or be mutated to have, low Δ15 desaturase activity. Alternatively, a host cell for Δ6 desaturase expression may have, or be mutated to have, high Δ12 desaturase activity.

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, integration of constructs can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs

are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

When increased expression of the desaturase polypeptide in the source plant is desired, several methods can be employed. Additional genes encoding the desaturase polypeptide can be introduced into the host organism. Expression from the native desaturase locus also can be increased through homologous recombination, for example by inserting a stronger promoter into the host genome to cause increased expression, by removing destabilizing sequences from either the mRNA or the encoded protein by deleting that information from the host genome, or by adding stabilizing sequences to the mRNA (*see* USPN 4,910,141 and USPN 5,500,365.)

When it is desirable to express more than one different gene, appropriate regulatory regions and expression methods, introduced genes can be propagated in the host cell through use of replicating vectors or by integration into the host genome. Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of replication. Each introduced construct, whether integrated or not, should have a different means of selection and should lack homology to the other constructs to maintain stable expression and prevent reassortment of elements among constructs. Judicious choices of regulatory regions, selection means and method of propagation of the introduced construct can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

Constructs comprising the gene of interest may be introduced into a host cell by standard techniques. These techniques include transfection, infection, holistic impact, electroporation, microinjection, scraping, or any other method which introduces the gene of interest into the host cell (*see* USPN 4,743,548, USPN 4,795,855, USPN 5,068,193, USPN 5,188,958, USPN 5,463,174, USPN 5,565,346 and USPN 5,565,347). For convenience, a host cell which has been manipulated by any method to take up a DNA sequence or construct will be referred to as "transformed" or "recombinant" herein. The subject host will have at least have one copy of the expression construct and may have two or more, depending upon

whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy numbers.

The transformed host cell can be identified by selection for a marker contained on the introduced construct. Alternatively, a separate marker construct may be introduced with the desired construct, as many transformation techniques introduce many DNA molecules into host cells. Typically, transformed hosts are selected for their ability to grow on selective media. Selective media may incorporate an antibiotic or lack a factor necessary for growth of the untransformed host, such as a nutrient or growth factor. An introduced marker gene therefor may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when expressed in the transformed host cell. Desirably, resistance to kanamycin and the amino glycoside G418 are of interest (*see* USPN 5,034,322). Selection of a transformed host can also occur when the expressed marker protein can be detected, either directly or indirectly. The marker protein may be expressed alone or as a fusion to another protein. The marker protein can be detected by its enzymatic activity; for example β galactosidase can convert the substrate X-gal to a colored product, and luciferase can convert luciferin to a light-emitting product. The marker protein can be detected by its light-producing or modifying characteristics; for example, the green fluorescent protein of *Aequorea victoria* fluoresces when illuminated with blue light. Antibodies can be used to detect the marker protein or a molecular tag on, for example, a protein of interest. Cells expressing the marker protein or tag can be selected, for example, visually, or by techniques such as FACS or panning using antibodies.

The PUFAs produced using the subject methods and compositions may be found in the host plant tissue and/or plant part as free fatty acids or in conjugated forms such as acylglycerols, phospholipids, sulfolipids or glycolipids, and may be extracted from the host cell through a variety of means well-known in the art. Such means may include extraction with organic solvents, sonication, supercritical fluid extraction using for example carbon dioxide, and physical means such as presses, or combinations thereof. Of particular interest is extraction with hexane or methanol and chloroform. Where desirable, the aqueous layer can be acidified to

protonate negatively charged moieties and thereby increase partitioning of desired products into the organic layer. After extraction, the organic solvents can be removed by evaporation under a stream of nitrogen. When isolated in conjugated forms, the products are enzymatically or chemically cleaved to release the free fatty acid or a less complex conjugate of interest, and are then subjected to further manipulations to produce a desired end product. Desirably, conjugated forms of fatty acids are cleaved with potassium hydroxide.

PURIFICATION OF FATTY ACIDS

If further purification is necessary, standard methods can be employed. Such methods include extraction, treatment with urea, fractional crystallization, HPLC, fractional distillation, silica gel chromatography, high speed centrifugation or distillation, or combinations of these techniques. Protection of reactive groups, such as the acid or alkenyl groups, may be done at any step through known techniques, for example alkylation or iodination. Methods used include methylation of the fatty acids to produce methyl esters. Similarly, protecting groups may be removed at any step. Desirably, purification of fractions containing ARA, DHA and EPA is accomplished by treatment with urea and/or fractional distillation.

USES OF FATTY ACIDS

The uses of the fatty acids of subject invention are several. Probes based on the DNAs of the present invention may find use in methods for isolating related molecules or in methods to detect organisms expressing desaturases. When used as probes, the DNAs or oligonucleotides need to be detectable. This is usually accomplished by attaching a label either at an internal site, for example via incorporation of a modified residue, or at the 5' or 3' terminus. Such labels can be directly detectable, can bind to a secondary molecule that is detectably labeled, or can bind to an unlabelled secondary molecule and a detectably labeled tertiary molecule; this process can be extended as long as is practical to achieve a satisfactorily detectable signal without unacceptable levels of background signal. Secondary, tertiary, or bridging systems can include use of antibodies directed against any other molecule, including labels or other antibodies, or can involve any

molecules which bind to each other, for example a biotin-streptavidin/avidin system. Detectable labels typically include radioactive isotopes, molecules which chemically or enzymatically produce or alter light, enzymes which produce detectable reaction products, magnetic molecules, fluorescent molecules or molecules whose fluorescence or light-emitting characteristics change upon binding. Examples of labelling methods can be found in USPN 5,011,770. Alternatively, the binding of target molecules can be directly detected by measuring the change in heat of solution on binding of probe to target via isothermal titration calorimetry, or by coating the probe or target on a surface and detecting the change in scattering of light from the surface produced by binding of target or probe, respectively, as may be done with the BIAcore system.

The invention will be better understood by reference to the following non-limiting examples.

Examples

Example 1

Expression of ω-3 desaturase from *C. elegans* in transgenic plants.

The D15/ω-3 activity of *Brassica napus* can be increased by the expression of an ω-3 desaturase from *C. elegans*. The fat-1 cDNA clone (Genbank accession L41807; Spychalla, J. P., Kinney, A. J., and Browse, J. 1997 P.N.A.S. 94, 1142-1147 was obtained from John Browse at Washington State University. The fat-1 cDNA was modified by PCR to introduce cloning sites using the following primers:

Fat-1forward:

5'-CUACUACUACUACTGCAGACAATGGTCGCTCATTCCTCAGA-3'

Fat-1reverse:

5'- CAUCAUCAUCAUGCGGCCGCTTACTTGGCCTTTGCCTT - 3'

These primers allowed the amplification of the entire coding region and added PstI and NotI sites to the 5'- and 3'-ends, respectively. The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL)

to create pCGN5562. The sequence was verified by sequencing of both strands to be sure no changes were introduced by PCR. For seed specific expression, the Fat-1 coding region was cut out of pCGN5562 as a PstI/NotI fragment and inserted between the PstI/NotI sites of the binary vector, pCGN8623, to create pCGN5563. PCGN5563 can be introduced into Brassica napus via *Agrobacterium*-mediated transformation.

Construction of pCGN8623

The polylinker region of the napin promoter cassette, pCGN7770, was replaced by ligating the following oligonucleotides:

5'- TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC -3' and

5'- TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3'. These oligonucleotides were ligated into SalI/XhoI-digested pCGN7770 to produce pCGN8619. These oligos encode BamHI, NotI, HindIII, and PstI restriction sites. pCGN8619 contains the oligos oriented such that the PstI site is closest to the napin 5' regulatory region. A fragment containing the napin 5' regulatory region, polylinker, and napin 3' region was removed from pCGN8619 by digestion with Asp718I. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8623.

To produce high levels of stearidonic acid in *Brassica*, the C. elegans ω-3 desaturase can be combined with D6- and D12-desaturases from *Mortierella alpina*. PCGN5563-transformed plants may be crossed with pCGN5544-transformed plants expressing the D6-and D12-desaturases.

The resulting F1 seeds can be analyzed for stearidonic acid content and selected F1 plants can be used for self-pollination to produce F2 seed, or as donors for production of dihaploids, or additional crosses.

An alternative method to combine the fat-1 cDNA with *M. alpina* D6 and D12 desaturases is to combine them on one T-DNA for transformation. The fat-1 coding region from pCGN5562 can be cut out as a PstI/NotI fragment and inserted into PstI/NotI digested pCGN8619. The transcriptional unit consisting of the napin 5' regulatory region, the fat-1 coding region, and the napin 3'-regulatory region can be cut out as a Sse8387I fragment and inserted into pCGN5544 cut with Sse8387I. The resulting plasmid would contain three napin transcriptional units containing the *C. elegans* ω-3 desaturase, *M. alpina* D6 desaturase, and *M. alpina* D12 desaturase, all oriented in the same direction as the 35S/nptII/tml transcriptional unit used for selection of transformed tissue.

Example 2

Over-Expression of D15-desaturase Activity in Transgenic Canola

The D15-desaturase activity of Brassica napus can be increased by over-expression of the D15-desaturase cDNA clone.

A *B. napus* D15-desaturase cDNA clone was obtained by PCR amplification of first-strand cDNA derived from *B. napus* cv. 212/86. The primers were based on published sequence: Genbank # L01418 Arondel et al, 1992 Science 258:1353-1355.

The following primers were used:

Bnd15-FORWARD

5'-CUACUACUACUAGAGCTCAGCGATGGTTGTTGCTATGGAC-3'

Bnd15-REVERSE

5'-CAUCAUCAUCAUGAATTCTTAATTGATTTTAGATTTG-3'

These primers allowed the amplification of the entire coding region and added SacI and EcoRI sites to the 5'- and 3'-ends, respectively

The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5520. The sequence was verified by sequencing of both strands to be sure that the open reading frame remained intact.

For seed specific expression, the D15-desaturase coding region was cut out of pCGN5520 as a BamHI/SalI fragment and inserted between the BglII and XhoI sites of the pCGN7770, to create pCGN5557. The PstI fragment of pCGN5557 containing the napin 5'-regulatory region, *B. napus* D15-desaturase, and napin 3'-regulatory region was inserted into the PstI site of the binary vector, pCGN5138 to produce pCGN5558. pCGN5558 was introduced into *Brassica napus* via *Agrobacterium*-mediated transformation.

To produce high levels of stearidonic acid in *Brassica*, the D15-desaturase can be combined with D6- and D12-desaturases from *Mortierella alpina*. PCGN5558-transformed plants may be crossed with pCGN5544-transformed plants expressing the D6 and D12-desaturases. The resulting F1 seeds can be analyzed for stearidonic acid content and selected F1 plants can be used for self-pollination to produce F2 seed, or as donors for production of dihaploids, or additional crosses.

An alternative method to combine the *B. napus* D15-desaturase with *M. alpina* D6 and D12 desaturases is to combine them on one T-DNA for transformation. The transcription cassette consisting of the napin 5'-regulatory region, the D15-desaturase coding region, and the napin 3'-regulatory region can be cut out of pCGN5557 as a SwaI fragment and inserted into SwaI-digested pCGN5544. The resulting plasmid would contain three napin transcriptional units containing the *M. alpina* D6 desaturase, the *B. napus* D15-desaturase, and the *M. alpina* D12 desaturase, all oriented in the same direction as the 35S/nptII/tml transcriptional unit used for selection of transformed tissue.

Example 3

Expression of Δ5 Desaturase in Plants

Expression in Leaves

Ma29 is a putative *M. alpina* D5 desaturase as determined by sequence homology. This experiment was designed to determine whether leaves expressing Ma29 (as determined by Northern) were able to convert exogenously applied DGLA (20:3) to ARA (20:4).

The Ma29 desaturase cDNA was modified by PCR to introduce convenient restriction sites for cloning. The desaturase coding region has been inserted into a d35 cassette under the control of the double 35S promoter for expression in *Brassica* leaves (pCGN5525) following standard protocols (*see* USPN 5,424,200 and USPN 5,106,739). Transgenic *Brassica* plants containing pCGN5525 were generated following standard protocols (*see* USPN 5,188,958 and USPN 5,463,174).

In the first experiment, three plants were used: a control, LP004-1, and two transgenics,, 5525-23 and 5525-29. LP004 is a low-linolenic *Brassica* variety. Leaves of each were selected for one of three treatments: water, GLA or DGLA. GLA and DGLA were purchased as sodium salts from NuChek Prep and dissolved in water at 1 mg/ml. Aliquots were capped under N_2 and stored at -70 degrees C. Leaves were treated by applying a 50 µl drop to the upper surface and gently spreading with a gloved finger to cover the entire surface. Applications were made approximately 30 minutes before the end of the light cycle to minimize any photo-oxidation of the applied fatty acids. After 6 days of treatment one leaf from each treatment was harvested and cut in half through the mid rib. One half was washed with water to attempt to remove unincorporated fatty acid. Leaf samples were lyophilized overnight, and fatty acid composition determined by gas chromatography (GC). The results are shown in Table 1.

Table 1

Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants

Treatment	SPL	16:00	16:01	18:00	18:01	18:1e	18:1v	18:02	18:3g	18:03	18:04	20:00	20:01
	#	%	%	%	%	%	%	%	%	%	%	%	%
Water	33	12.95	0.08	2.63	2.51	1.54	0.98	16.76	0	45.52	0	0.09	0
	34	13.00	0.09	2.67	2.56	1.55	1.00	16.86	0	44.59	0	0.15	0
	35	14.13	0.09	2.37	2.15	1.27	0.87	16.71	0	49.91	0	0.05	0.01
	36	13.92	0.08	2.32	2.07	1.21	0.86	16.16	0	50.25	0	0.05	0
	37	13.79	0.11	2.10	2.12	1.26	0.86	15.90	0.08	46.29	0	0.54	0.01
	38	12.80	0.09	1.94	2.08	1.35	0.73	14.54	0.11	45.61	0	0.49	0.01
GLA	39	12.10	0.09	2.37	2.10	1.29	0.82	14.85	1.63	43.66	0	0.53	0
	40	12.78	0.10	2.34	2.22	1.36	0.86	15.29	1.72	47.22	0	0.50	0.02
	41	13.71	0.07	2.68	2.16	1.34	0.82	15.92	2.12	46.55	0	0.09	0
	42	14.10	0.07	2.75	2.35	1.51	0.84	16.66	1.56	46.41	0	0.09	0.01
	43	13.62	0.09	2.22	1.94	1.21	0.73	14.68	2.42	46.69	0	0.51	0.01
	44	13.92	0.09	2.20	2.17	1.32	0.85	15.22	2.30	46.05	0	0.53	0.02
DGLA	45	12.45	0.14	2.30	2.28	1.37	0.91	15.65	0.07	44.62	0	0.12	0.01
	46	12.67	0.15	2.69	2.50	1.58	0.92	15.96	0.09	42.77	0	0.56	0.01
	47	12.56	0.23	3.40	1.98	1.13	0.86	13.57	0.03	45.52	0	0.51	0.01
	48	13.07	0.24	3.60	2.51	1.63	0.88	13.54	0.04	45.13	0	0.50	0.01
	49	13.26	0.07	2.81	2.34	1.67	0.67	16.04	0.04	43.89	0	0.59	0
	50	13.53	0.07	2.84	2.41	1.70	0.70	16.07	0.02	44.90	0	0.60	0.01

Table 1 - Continued
Fatty Acid Analysis of Leaves from Ma29 Transgenic *Brassica* Plants

Treatment	SPT.	20:02	20:03	20:04	20:05	22:00	22:01	22:02	22:03	22:06	24:0	24:1
	#	%	%	%	%	%	%	%	%	%	%	%
Water	33	0	0	0.29	0	0.01	0.09	16.26	0	0	0.38	0.18
	34	0.01	0	0.26	0	0.14	0.10	16.82	0.02	0.05	0.36	0.27
	35	0.01	0	0.25	0	0.12	0.06	11.29	0.04	0.05	0.29	0.25
	36	0	0.01	0.26	0	0.07	0.04	11.82	0.03	0.36	0.28	0.21
	37	0.02	0	0.21	0	0.18	0.08	15.87	0.06	0.20	0.30	0.17
	38	0.01	0	0.24	0	0.15	0.07	13.64	0.09	0.08	5.89	0.23
GLA	39	0.02	0.01	0.27	0	0.10	0.08	16.25	3.42	0.19	0.37	0.17
	40	0.01	0	0.27	0	0.10	0.10	14.74	0.05	0.10	0.36	0.14
	41	0	0	0.27	0	0.20	0.10	13.15	0.13	0.29	0.33	0.20
	42	0	0	0.28	0	0.11	0.11	12.60	0.02	0.24	0.38	0.13
	43	0.01	0	0.28	0	0.10	0.03	14.73	0.01	0.24	0.34	0.14
	44	0.02	0	0.26	0	0.13	0.07	14.43	0.05	0.16	0.33	0.17
DGLA	45	0.06	1.21	0.26	0	0.07	0.07	18.67	0.02	0.21	0.36	0.13
	46	0	1.94	0.27	0	0.11	0.09	17.97	0.09	0.39	0.41	0.11
	47	0.01	0.69	0.96	0	0.11	0.07	17.96	0	0.22	0.49	0.20
	48	0.01	0.70	0.74	0	0.14	0.09	17.14	0.05	0.32	0.52	0.10
	49	0	0.35	1.11	0	0.10	0.07	17.26	0.07	0.23	0.39	0.18
	50	0	0.20	0.87	0	0.21	0.07	15.73	0.04	0.15	0.37	0.18

Leaves treated with GLA contained from 1.56 to 2.4 wt% GLA. The fatty acid analysis showed that the lipid composition of control and transgenic leaves was essentially the same. Leaves of control plants treated with DGLA contained 1.2-1.9 w% DGLA and background amounts of ARA (.26-.27 wt%). Transgenic leaves contained only .2-.7 wt% DGLA, but levels of ARA were increased (.74-1.1 wt%) indicating that the DGLA was converted to ARA in these leaves.

Expression in Seed

The purpose of this experiment was to determine whether a construct with the seed specific napin promoter would enable expression in seed.

The Ma29 cDNA was modified by PCR to introduce *Xho*I cloning sites upstream and downstream of the start and stop codons, respectively, using the following primers:

Madxho-forward:

5'-CUACUACUACUACTCGAGCAAGATGGGAACGGACCAAGG

Madxho-reverse:

5'-CAUCAUCAUCAUCTCGAGCTACTCTTCCTTGGGACGGAG

The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5522 and the Δ5 desaturase sequence was verified by sequencing of both strands.

For seed-specific expression, the Ma29 coding region was cut out of pCGN5522 as an *Xho*I fragment and inserted into the *Sal*I site of the napin expression cassette, pCGN3223, to create pCGN5528. The *Hin*dIII fragment of pCGN5528 containing the napin 5' regulatory region, the Ma29 coding region, and the napin 3' regulatory region was inserted into the *Hin*dIII site of pCGN1557 to create pCGN5531. Two copies of the napin transcriptional unit were inserted in tandem. This tandem construct can permit higher expression of the desaturases per genetic loci. pCGN5531 was introduced into *Brassica napus* cv.LP004 via Agrobacterium mediated transformation.

The fatty acid composition of twenty-seed pools of mature T2 seeds was analyzed by GC. Table 2 shows the results obtained with independent transformed lines as compared to non-transformed LP004 seed. The transgenic seeds containing

pCGN5531 contain two fatty acids that are not present in the control seeds, tentatively identified as taxoleic acid (5,9-18:2) and pinolenic acid (5,9,12-18:3), based on their elution relative to oleic and linoleic acid. These would be the expected products of Δ5 desaturation of oleic and linoleic acids. No other differences in fatty acid composition were observed in the transgenic seeds.

Example 4

Production of D5-desaturated Fatty Acids in Transgenic Plants

The construction of pCGN5531 (D5-desaturase) and fatty acid composition of T2 seed pools is described in Example 3. This example takes the seeds through one more generation and discusses ways to maximize the D5-desaturated fatty acids.

Example 3 describes the fatty acid composition of T2 seed pools of pCGN5531-transformed *B. napus* cv. LP004 plants. To investigate the segregation of D5-desaturated fatty acids in the T2 seeds and to identify individual plants to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Results of some of these analyses are shown in the accompanying Table 3. D5,9-18:2 accumulated to as high as 12% of the total fatty acids and D5,9,12-18:3 accumulated to up to 0.77% of the fatty acids. These and other individually selected T2 plants were grown in the greenhouse to produce T3 seed.

Table 2
Composition of T2 Pooled Seed

	16:0 %	16:1 %	18:0 %	18:1 %	(5,9)18:2 %	18:2 %	(5,9,12)18:3 %	18:3 %	20:0 %	20:1 %	20:2 %	22:0 %	22:1 %	24:0 %
LP(X)4 control	3.86	0.15	3.05	69.1	0	18.51	0.01	1.65	1.09	1.40	0.03	0.63	0.05	0.42
5531-1	4.26	0.15	3.23	62.33	4.07	21.44	0.33	1.38	0.91	1.04	0.05	0.41	0.03	0.27
5531 2	3.78	0.14	3.37	66.18	4.57	17.31	0.27	1.30	1.03	1.18	0	0.47	0.01	0.30
5531-6	3.78	0.13	3.47	63.61	6.21	17.97	0.38	1.34	1.04	1.14	0.05	0.49	0.02	0.26
5531-10	3.96	0.17	3.28	63.82	5.41	18.58	0.32	1.43	0.98	1.11	0.02	0.50	0	0.31
5531-16	3.91	0.17	3.33	64.31	5.03	18.98	0.33	1.39	0.96	1.11	0	0.44	0	0
5531-28	3.81	0.13	2.58	62.64	5.36	20.95	0.45	1.39	0.83	1.15	0.01	0.36	0.05	0.21

Table 3

Fatty acid analysis of selected T2 half-seeds from pCGN5531-LP004 events

CYCLE ID	SPL NO	STRAIN ID	12:0	14:0	16:0	16:1	18:0	18:1	18:2 Δ5,9	18:2 Δ9,12	18:3 Δ5,9,12	18:3 Δ9,12,15
97XX1539	93	5531-LP004-6	0.03	0.07	3.92	0.17	3.5	61.32	12.22	15.36	0.77	1.36
97XX1539	29	5531-LP004-6	0.01	0.04	3.6	0.09	3.23	63.77	10.63	14.47	0	1.22
97XX1539	38	5531-LP004-6	0.01	0.05	3.71	0.09	3.02	65.13	10.57	13.98	0	1.06
97XX1539	41	5531-LP004-6	0.01	0.05	3.64	0.07	3.22	62.51	9.7	16.63	0	1.28
97XX1539	18	5531-LP004-6	0.02	0.06	3.69	0.09	3.36	63.79	9.63	15.29	0.63	1.15
97XX1539	85	5531-LP004-6	0.01	0.06	3.6	0.09	3.54	64.81	9.54	13.69	0.6	1.26
98GC0023	98	5531-LP004-23	0.01	0.05	3.5	0.09	3.12	64.97	9.92	13.62	0.55	1.25
98GC0023	32	5531-LP004-23	0.01	0.05	3.43	0.08	2.62	65.21	9.83	14.28	0.59	1.15
98GC0023	78	5531-LP004-23	0.01	0.05	3.45	0.07	2.78	64.97	9.34	14.69	0.58	1.17
98GC0023	86	5531-LP004-23	0.01	0.05	3.32	0.08	2.7	64.18	9.08	15.99	0.68	1.18
98GC0023	67	5531-LP004-23	0.01	0.04	3.49	0.08	3.03	64.14	8.78	15.95	0.62	1.08
98GC0023	52	5531-LP004-23	0.01	0.03	3.38	0.07	2.56	67.44	8.65	13.55	0.5	1.02

To maximize the accumulation of D5,9 18:2 in seed oil, the pCGN5531 construct could be introduced into a high oleic acid variety of canola. A high-oleic variety could be obtained by mutation, so-suppression, or antisense suppression of the D12 and D15 desaturases or other necessary co-factors.

To maximize accumulation of D5,9,12 18:3 in canola, the pCGN5531 construct could be introduced into a high linoleic strain of canola. This could be achieved by crossing pCGN5531-transformed plants with pCGN5542-(M. alpina D12-desaturase) transformed plants. Alternatively, the D5 and D12 desaturases could be combined on one T-DNA for transformation. The transcriptional unit consisting of the napin 5'-regulatory region, the *M. alpina* D12-desaturase coding region, and the napin 3'-regulatory region can be cut out of pCGN5541 (described in CGAB320) as a NotI fragment. NotI/XbaI linkers could be ligated and the resulting fragment inserted into the XbaI site of pCGN5531. The resulting plasmid would contain three napin transcriptional units containing the *M. alpina* D12 desaturase, and two copies of the napin/*M. alphina* D5 desaturase/napin unit, all oriented in the same direction as the 35S/nptII/tml transcriptional unit used for selection of transformed tissue.

Example 5

Expression of *M. alpina* Δ6 Desaturase in *Brassica napus*

A nucleic acid sequence from a partial cDNA clone, Ma524, encoding a Δ6 fatty acid desaturase from *Mortierella alpina* was obtained by random sequencing of clones from the *M. alpina* cDNA library. The Ma524 cDNA was modified by PCR to introduce cloning sites using the following primers:

Ma524PCR-1

5'-CUACUACUACUATCTAGACTCGAGACCATGGCTGCTGCT CCAGTGTG

Ma524PCR-2

5'-CAUCAUCAUCAUAGGCCTCGAGTTACTGCGCCTTACCCAT

These primers allowed the amplification of the entire coding region and added *Xbal* and *Xhol* sites to the 5'-end and *Xhol* and *Stul* sites to the 3' end. The PCR product was subcloned into pAMP1 (GIBCOBRL) using the CloneAmp system (GIBCOBRL) to create pCGN5535 and the Δ6 desaturase sequence was verified by sequencing of both strands.

For seed-specific expression, the Ma524 coding region was cut out of pCGN5535 as an *Xhol* fragment and inserted into the *Sall* site of the napin expression cassette, pCGN3223, to create pCGN5536. The *Notl* fragment of pCGN5536 containing the napin 5' regulatory region, the Ma524 coding region, and the napin 3' regulatory region was inserted into the *Notl* site of pCGN1557 to create pCGN5538. pCGN5538 was introduced into *Brassica napus* cv.LP004 via *Agrobacterium* mediated transformation.

Maturing T2 seeds were collected from 6 independent transformation events in the greenhouse. The fatty acid composition of single seeds was analyzed by GC. Table 4 shows the results of control LP004 seeds and six 5538 lines. All of the 5538 lines except #8 produced seeds containing GLA. Presence of GLA segregated in these seeds as is expected for the T2 selfed seed population. In addition to GLA, the *M. alpina* Δ6 desaturase is capable of producing 18:4 (stearidonic) and another fatty acid believed to be the 6,9-18:2.

The above results show that desaturases with three different substrate specificities can be expressed in a heterologous system and used to produce poly-unsaturated long chain fatty acids. Exemplified were the production of ARA (20:4) from the precursor 20:3 (DGLA), the production of GLA (18:3) from 18:2 substrate, and the conversion of 18:1 substrate to 18:2, which is the precursor for GLA.

Table 4

Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #		16:0 %	16:1 %	18:0 %	18:1	6,9 18:2 %	18:2 %	18:3ga %	18:3 %	18:4 %	20:1 %	22:0 %	22:1 %	24:0 %	24:1 %
	LPO04-1	4.33	0.21	3.78	72.49	0	13.97	0	1.7	0	1.34	0.71	0.02	0.58	0.27
	-2	4.01	0.16	3.09	73.59	0	14.36	0.01	1.4	0	1.43	0.66	0.02	0.5	0.2
	-3	4.12	0.19	3.56	70.25	0	17.28	0	1.57	0	1.28	0.5	0.02	0.39	0.2
	-4	4.22	0.2	2.7	70.25	0	17.86	0	1.61	0	1.31	0.53	0.02	0.4	0.24
	-5	4.02	0.16	3.41	72.91	0	14.45	0.01	1.45	0	1.37	0.7	0.02	0.51	0.26
	-6	4.22	0.18	3.23	71.47	0	15.92	0.01	1.52	0	1.32	0.69	0.02	0.51	0.27
	-7	4.1	0.16	3.47	72.06	0	15.23	0	1.52	0	1.32	0.63	0.03	0.49	0.23
	-9	4.01	0.17	3.71	72.98	0	13.97	0.01	1.41	0	1.45	0.74	0.03	0.58	0.23
	-10	4.04	0.16	3.57	70.03	0	17.46	0	1.5	0	1.33	0.61	0.03	0.36	0.24
	5538-1-1	4.61	0.2	3.48	68.12	1.37	10.68	7.48	1.04	0.33	1.19	0.49	0.02	0.33	0.13
	-2	4.61	0.22	3.46	68.84	1.36	10.28	7.04	1.01	0.31	1.15	0.48	0.02	0.39	0
	-3	4.78	0.24	3.24	65.86	0	21.36	0	1.49	0	1.08	0.46	0.02	0.38	0.22
	-4	4.84	0.3	3.89	67.64	1.67	9.9	6.97	1.02	0.36	1.14	0.53	0.02	0.5	0.18
	-5	4.64	0.2	3.58	64.5	3.61	8.85	10.14	0.95	0.48	1.19	0.47	0.01	0.33	0.12
	-6	4.91	0.27	3.44	66.51	1.48	11.14	7.74	1.15	0.33	1.08	0.49	0.02	0.34	0.13
	-7	4.67	0.22	3.24	65.78	1.27	11.92	8.38	1.2	0	1.12	0.47	0.02	0.37	0.16
	-8	4.59	0.22	3.4	70.77	0	16.71	0	1.35	0	1.14	0.48	0.02	0.39	0.15
	-9	4.63	0.23	3.51	69.66	2.01	8.77	7.24	0.97	0	1.18	0.52	0.02	0.3	0.11
	-10	4.56	0.19	3.55	70.68	0	16.89	0	1.37	0	1.22	0.54	0.02	0.22	0.03
	5538-3-1	4.74	0.21	3.43	67.52	1.29	10.91	7.77	1.03	0.28	1.11	0.5	0.02	0.35	0.14
	-2	4.72	0.21	3.24	67.42	1.63	10.37	8.4	0.99	0	1.12	0.49	0.02	0.36	0.15
	-3	4.24	0.21	3.52	71.31	0	16.53	0	1.33	0	1.12	0.45	0.02	0.4	0.14

Table 4

Fatty Acid Analysis of Seeds from MaS24 Transgenic *Brassica* Plants

SPL #		16:0 %	16:1 %	18:0 %	18:1	6,9 18:2 %	18:2 %	18:3ga %	18:3 %	18:4 %	20:1 %	22:0 %	22:1 %	24:0 %	24:1 %
	-4	4.64	0.21	3.45	67.92	1.65	9.91	7.97	0.91	0.33	1.14	0.47	0.02	0.37	0.14
	-5	4.91	0.25	3.31	67.19	0	19.92	0.01	1.39	0	1.05	0.48	0.02	0.37	0.14
	-6	4.67	0.21	3.25	67.07	1.23	11.32	8.35	0.99	0	1.16	0.47	0.02	0.33	0.16
	-7	4.53	0.19	2.94	64.8	4.94	8.45	9.95	0.93	0.44	1.13	0.37	0.01	0.27	0.12
	-8	4.66	0.22	3.68	67.33	0.71	12	6.99	1.1	0.24	1.18	0.48	0.03	0.36	0.17
	-9	4.65	0.24	3.11	67.42	0.64	12.71	6.93	1.16	0.25	1.08	0.45	0.02	0.32	0.17
	-10	4.88	0.27	3.33	65.75	0.86	12.89	7.7	1.1	0.24	1.08	0.46	0 01	0.34	0.16
	5538-4-1	4.65	0.24	3.8	62.41	0	24.68	0	1.6	0.01	0.99	0.45	0.02	0.33	0.13
	-2	5.37	0.31	3	57.98	0.38	18.04	10.5	1.41	0	0.99	0.48	0.02	0.3	0.19
	-3	4.61	0.22	3.07	63.62	0.3	16.46	7.67	1.2	0	1.18	0.45	0.02	0.29	0.14
	-4	4.39	0.19	2.93	65.97	0	22.36	0	1.45	0	1.17	0.41	0.03	0.32	0.15
	-5	5.22	0.29	3.85	62.1	2.35	10.25	11.39	0.93	0 41	1.04	0.6	0.02	0.47	0.17
	-6	4.66	0.18	2.85	66.79	0.5	13.03	7.66	0.97	0.22	1.28	0.42	0.02	0.31	0.14
	-7	4.85	0.26	3.03	57.43	0.26	28.04	0.01	2.59	0.01	1.13	0.56	0.02	0.4	0.23
	-8	5.43	0.28	2.94	54.8	1.84	13.79	15.67	1.36	0.53	1.1	0.55	0.02	0.35	0.19
	-9	4.88	0.24	3.32	62.3	0.58	14.86	9.04	1.34	0.29	1.13	0.52	0.02	0.37	0.19
	-10	4.53	0.2	2.73	64.2	0.07	24.15	0	1.52	0	1.09	0.39	0.02	0.27	0.17
	5538-5-1	4.5	0.15	3.35	66.71	0.88	11.7	8.38	1.04	0.3	1.24	0.49	0.02	0.29	0.17
	-2	4.77	0.23	3.06	62.67	0.68	15.2	8.8	1.31	0.28	1.15	0.46	0.02	0.3	0.19
	-3	4.59	0.22	3.61	64.35	2.29	9.95	10.57	1.01	0.45	1.21	0.48	0.02	0.26	0.16
	-4	4.86	0.26	3.4	67.69	0.65	12.24	6.61	1.09	0.23	1.07	0.45	0.02	0.32	0.14
	-5	4.49	0.21	3.3	69.25	0.04	16.51	2.18	1.2	0	1.11	0.44	0.02	0.33	0.16

Table 4

Fatty Acid Analysis of Seeds from Ma524 Transgenic *Brassica* Plants

SPL #		16:0 %	16:1 %	18:0 %	18:1	6,9 18:2 %	18:2 %	18:3ga %	18:3 %	18:4 %	20:1 %	22:0 %	22:1 %	24:0 %	24:1 %
	-6	4.5	0.21	3.47	70.48	0.08	14.9	2.19	1.22	0	1.13	0.49	0.02	0.33	0.16
	-7	4.39	0.21	3.44	67.59	2.38	9.24	8.98	0.89	0	1.18	0.44	0.02	0.28	0.14
	-8	4.52	0.22	3.17	68.33	0.01	18.91	0.73	1.32	0.01	1.08	0.45	0.02	0.29	0.17
	-9	4.68	0.2	3.05	64.03	1.93	11.03	11.41	1.02	0.01	1.15	0.39	0.02	0.21	0.15
	-10	4.57	0.2	3.1	67.21	0 61	12.62	7.68	1.07	0.25	1.14	0 43	0.02	0.25	0.15
	5538-8-1	4.95	0.26	3.14	64.04	0	23.38	0	1.54	0	0.99	0.42	0.02	0.38	0.17
	-2	4.91	0.26	3.71	62.33	0	23.97	0	1.77	0	0.95	0.53	0.02	0.42	0.19
	-3	4.73	0.25	4.04	63.83	0	22.36	0.01	1.73	0	1.05	0.55	0.02	0.45	0.16
	-4	5.1	0.35	3.8	60.45	0	24.45	0.01	2.13	0	1.07	0.65	0.03	0.53	0.24
	-5	4.98	0.3	3.91	62.48	0	23.44	0	1.77	0	1.01	0.51	0.01	0.43	0.21
	-6	4.62	0.21	3.99	66.14	0	20.38	0	1.48	0	1.15	0.53	0.02	0.48	0.19
	-7	4.64	0.22	3.55	64.6	0	22.65	0	1.38	0	1.09	0.45	0.02	0.41	0.19
	8	5.65	0.38	3.18	56.6	0	30.83	0.02	0.02	0	0.98	0.55	0.03	0.39	0.26
	-9	8.53	0.63	6.9	51.76	0	26.01	0	0.01	0	1.41	1.21	0.07	0.96	0.33
	-10	5.52	0.4	3.97	57.92	0	28.95	0	0.02	0	0.95	0.52	0.02	0.41	0.16
	5538 10 1	4.44	0.19	3.5	68.42	0	19.51	0	1.32	0	1.14	0.45	0.02	0.31	0.16
	-2	4.57	0.21	3.07	66.08	0	21.99	0.01	1.36	0	1.12	0.41	0.02	0.31	0.16
	-3	4.63	0.21	3.48	67.43	0	20.27	0.01	1.32	0	1.12	0.46	0.02	0.21	0.08
	-4	4.69	0.19	3.22	64.62	0	23.16	0	1.35	0	1.08	0.46	0.02	0.33	0.2
	-5	4.58	0.2	3.4	68.75	0	20.17	0.01	0.02	0	1.1	0.45	0.02	0.34	0.17
	-8	4.55	0.21	0	73.55	0.05	14.91	2.76	1.21	0.07	1.24	0.51	0.02	0.19	0
	-9	4.58	0.21	3.28	66.19	0	21.55	0	1.35	0	1.12	0.43	0.02	0.33	0.16

Table 4

Fatty Acid Analysis of Seeds from MS24 Transgenic *Brassica* Plants

			16:0	16:1	18:0	18:1	6,9 18:2	18:2	18:3ga	18:3	18:4	20:1	22:0	22:1	24:0	24:1
SPL			%	%	%		%	%	%	%	%	%	%	%	%	%
#																
		-10	4.52	0.2	3.4	68.37	0	19.33	0.01	1.3	0	1.13	0.46	0.02	0.35	0.18

Example 6

Production of D6,9 18:2 in Canola Oil

Example 5 described construction of pCGN5538 designed to express the *M. alpina* D6 desaturase in seeds of transgenic canola. Table 4 in that example showed examples of single seed analyses from 6 independent transgenic events. Significant amounts of GLA were produced, in addition to the D6,9 18:2 fatty acid.

A total of 29 independent pCGN5538-transformed transgenic plants of the low-linolenic LP004 cultivar were regenerated and grown in the greenhouse. Table 5 shows the fatty acid composition of 20-seed pools of T2 seed from each event. Seven of the lines contained more than 2% of the D6,9 18:2 in the seed pools. To identify and select plants with high amounts of D6,9 18:2 to be taken on to subsequent generations, half-seed analysis was done. Seeds were germinated overnight in the dark at 30 degrees on water-soaked filter paper. The outer cotyledon was excised for GC analysis and the rest of the seedling was planted in soil. Based on results of fatty acid analysis, selected T2 plants were grown in the greenhouse to produce T3 seed. The selection cycle was repeated; pools of T3 seed were analyzed for D6,9 18:2, T3 half-seeds were dissected and analyzed, and selected T3 plants were grown in the greenhouse to produce T4 seed. Pools of T4 seed were analyzed for fatty acid composition. Table 5 summarizes the results of this process for lines derived from one of the original transgenic events, 5538-LP004-25. Levels of D6,9 18:2 have thus been maintained through 3 generations.

To maximize the amount of D6,9 18:2 that could be produced, the pCGN5538 construct could be introduced into a high oleic acid variety of canola either by transformation or crossing. A high-oleic variety could be obtained by mutation, co-suppression, or antisense suppression of the D12 and D15 desaturases or other necessary co-factors.

Table 5

Fatty Acid Composition of 10-seed Pools of PCGN5538 T2 Seeds

								C9	6,9		C6912	C91215						
SPL	5538-LP004	12:0	14:0	16:0	16:1	18:0	18:1	18:1	18:2	18:2	18:3	18:3	18:4	20:0	20:1	20:2	22:0	22:2
#	event	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
	31	0.02	0.06	4.07	0.07	0	59.4		5.4	10.07	15.93	1.2	0.6	0.98	1.16	0.0	0.44	0.03
	29	0.01	0.05	3.81	0.14	0	60.7		4.53	10.9	14.77	1.03	0.55	1.09	1.26	0.0	0.46	0.02
	19	0.02	0.07	4.27	0.13	0	62.9		4.17	10.03	13.14	1.02	0.59	1.18	1.25	0.0	0.53	0.02
	14	0	0	5.29	0.24	3.8	49.1		1.02	23.44	11.21	2.26	0.34	1.45	0.93	0.0	0.76	0
	22	0.02	0.05	3.87	0.09	0	64.1		2.59	12.57	11.18	1.27	0.6	1.18	1.03	0.1	0.56	0
	9	0.01	0.06	4.57	0.16	0	62.9		3.4	12.05	11.15	1.27	0.6	1.28	1.18	0.0	0.56	0.03
	25	0.01	0.06	4.17	0.14	0	62.4		2.49	14.42	11.03	1.2	0.46	1.18	1.15	0.0	0.53	0.01
	15	0.01	0.05	3.94	0.11	0	65.2		2.08	12.77	10.9	1.04	0.43	1.1	1.24	0.0	0.48	0.01
	18	0	0.06	5.34	0.29	0	58.4		1.42	18.19	10.53	1.8	0.49	1.2	1	0.0	0.58	0.02
	20	0.01	0.04	3.95	0.1	0	65.6		1.31	13.83	10.22	1.09	0.39	1.06	1.3	0.0	0.46	0.01
	7	0.02	0.07	4.04	0.11	0	62.1		0.92	18.12	8.72	1.77	0.35	1.26	1.19	0.0	0.58	0
	11	0.01	0.06	4.23	0.17	0	62.9		1.6	17.19	8.58	1.48	0.38	1.46	1.03	0.0	0.49	0.01
	27	0.02	0	3.99	0.14	0	65.3		0.64	17.85	7.89	1.36	0.31	1.08	1.21	0.0	0	0
	2	0.01	0.05	4.02	0.14	0	66.4		1.2	15.74	7.58	1.22	0.32	1.06	1.19	0.0	0.45	0
	28	0.01	0.04	3.77	0.11	0	67.5		0.79	15.56	7.58	1.12	0.28	0.97	1.23	0.0	0.44	0
	3	0.01	0.05	3.96	0.12	0	68.5		1.81	13.23	7.44	1.1	0.35	1.12	1.21	0.0	0.46	0.01
	21	0.01	0.05	3.74	0.1	0	66.9		1.16	13.9	6.97	1.35	0.28	1.15	1.27	0.0	0.52	0
	5	0.01	0.04	3.81	0.12	0	69.1		0.74	14.58	6.95	1.14	0.28	1.06	1.18	0.0	0.45	0
	6	0	0	2.84	0	3.06	62.5		1.55	18.44	6.94	1.21	0.39	1.04	1.33	0.1	0	0
	4	0.01	0.05	3.88	0.11	0	66.9		0.64	16.21	6.89	1.52	0.31	1.09	1.21	0.0	0.5	0
	30	0.01	0.04	3.89	0.12	0	68.6		0.72	15.58	6.47	1.17	0.23	1.03	1.07	0.0	0.46	0
	16	0.02	0.05	3.75	0.13	0	70.4		0.91	13.56	6.39	1.13	0.28	1.04	1.2	0.0	0.44	0.01
	26	0.01	0	3.77	0.12	0	67.6		0	21.08	3.61	1.37	0.13	0.96	1.16	0.0	0	0

Table 5

Fatty Acid Composition of 24-seed Pools of PDGN5534 T2 Seeds

								C9	6.9		C6912	C91215						
	23	0	0	4.92	0.22	0	65.2		0	22.23	3	1.79	0.11	1.28	1.11	0.0	0	0
	24	0.01	0	3.84	0.13	0	68.4		0.36	21	2.09	1.53	0.08	1.06	1.27	0.0	0	0
	10	0.01	0	3.74	0.11	0	70.4		0	20.82	0.65	1.3	0.03	1.05	1.18	0.0	0.46	0
	12	0.01	0	3.83	0.12	0	69.9		0	21.61	0.34	1.34	0	1.06	1.12	0.0	0.47	0
	17	0.01	0	0	0.13	0	72.6		0	23.03	0.24	1.51	0	1.18	1.21	0.0	0	0
	8	0.01	0	4.54	0.2	0	64.9		0	25.65	0.22	1.94	0	1.38	1.01	0.0	0	0
	13	0.01	0	3.99	0.16	0	65.8		0	25.97	0	1.58	0	1.17	1.16	0.0	0	0
	LPOO4 control	0.01	0.04	3.46	0.09	0	69.9		0	21.95	0	1.37	0.01	0.9	1.25	0.0	0.42	0

Example 7

Identification of potentially useful D15/ω-3 desaturases from other organisms

To look for desaturases involved in PUFA production, cDNA libraries were constructed from total RNA isolated from *Schizochytrium* (unknown species - proprietary strain supplied by Kelco in San Diego). Plasmid-based cDNA libraries were constructed in pSPORT1 (GIBCO-BRL) following manufacturer's instructions using a commercially available kit (GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid sequences that encode putative desaturases were identified through BLAST search of the databases and comparison to known D12 and D15 sequences.

One clone was identified from the *Schizochytrium* library with homology to both D12 and D15 desaturases; it is called 81-53-A2. The DNA Sequence is presented as Seq ID NO:1. The corresponding peptide sequence is presented as SEQ ID NO: 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

APPLICANT: KNUTZON, DEBORAH et al.

(ii) TITLE OF INVENTION: POLY-UNSATURATED FATTY ACIDS IN PLANTS

(iii) NUMBER OF SEQUENCES:

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LIMBACH & LIMBACH L.L.P.
(B) STREET: 2001 FERRY BUILDING
(C) CITY: SAN FRANCISCO
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94111

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Microsoft Word

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/833,610
(B) FILING DATE: 11-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MICHAEL R. WARD

(B) REGISTRATION NUMBER: 38,351

(C) REFERENCE/DOCKET NUMBER: CGMO-100

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 433-4150

(B) TELEFAX: (415) 433-8716

(C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

```
CACGGAAGCA AGCCTTGACA TCCTTTGCCA ACATGTGCAA GGTCGAGACC AAGCAGCACG      60
CTGCGGCCAC CGCCGTCCAG GCACCGGAGC AGCAGCAGCA GCAGCAGCAG CAGTCGCAGC     120
AGTCGCACCA GCTGCCGTCC GCAGCGCAAG CCTCGGCGGG GGAAGTCCTG GAAAAACGAC     180
CCATTATCCA TGGCAAGCAC AACCCAGACC TGCCCACGCT CGGAGAGATT CGCGCCGCGG     240
TGCCCAAGCA CTGCTTCCAG CGCTCGCTCC TCACCAGTTC GCTGTATCTT CGGCGCGACC     300
TCGTCATGGC AACGATCCTG TTCTGCATGG CGCGGCACTT CCTGCCCGTA TACGACATGG     360
GCGTCTTGGG CGCCATCGGC TGGACAAGCC TACGTAATTG TTCAAGGGAC AGTCTTTGCT     420
CCACTCTGGG TCCTCGGTCA TGAGTGCGGA CACCAGGCAT TTTCCAACTA CAGAGTAGTC     480
AACGACACCG TCGGCTACCT TGTGCACACT GCCTTGCTTG TGCCTTACTT TAGCTGGGCG     540
TACACGCATG GCTTGCACCA CGCCCGTGTC AACCACATGC TCGACGGCGA GTCTCACACT     600
CCCAACCTGC AAAAGAAAGT CATGGCTAAC TTTCAAAAGT TAGCCGACCT CATGGGCGAC     660
GAGGCCTTTG CTGTCCTCCA CGTCTTTGTT TATCTCCTCC TTCCGTGGCC GCTGTATATC     720
ATCAATGGCA GCGGGGCATC CAAGCGCAAC CACGAGGGTA AGCGGTGGTC AAAGGAGATG     780
GTCAAGCGGC CTAACCACTT CTTGCCCACC TCGGAGCTCT TCCGGGACAA GATGCGTCTC     840
AGTGTGGCAG GCTCTACGGC CGGTGTCCTC GTCGTCATTG CTAGCTTGTG TTACTGGGGT     900
TCCATCGAGG GTTCGCGGAC CGTGCTGCTT CAGTACTTTC TCCCTTACCT TGTTGTGAAC     960
GCCTACCTCA TTGGTTTCAC ATGGATGCAG CATACTCACC AAGATGTCCC GCATCTTGGC    1020
GAAGACGAAG TGGTCCTGGC TCGCTGGAAC CATTCTCACC ATCCATCGCC TTATCCTGCC    1080
TTTATCGACG TACTCACACA CCGCATCGGA TCCACGCACG TTGCGCATCA TCTTTTCTCG    1140
AAGATGCCCT GGTACCATGC CCGCGAAGCC ACTGTTCACA TCAAGGCTTT GCTTGAGCCC    1200
AAGGGGGTCT ACAACTATGA CCCGACGCCG TTTACAAGGC GCTGTACAAC ACCGCCAGAT    1260
ACTGTCACTT TATGGAGCGC GTCGAAGGCA TTCAGTTCTT CAAACACGTT CACGCTCAAT    1320
CTACAAAAGC AAAGGTCCTC TAAGATTTCC TCCTTCTAGG ACAATCACTT TCATTGCTAC    1380
CATACAATAT AACTTCATCG CCCCTTCCCG TAATCAATTT GTCTCTCTTT TC            1432
```

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:

(B) TYPE: aminoacid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(2) INFORMATION FOR SEQ ID NO:2:

MASTTQTCPRSERFAPRCPSTASSARSSPVRCILGATSSWQRSCSAWRGTSCPYTTWASWAPSAGQAYVIVQGTV
FAGLWVLGHECGHQAPSNYRVVNDTVGYLVHTALLVPYFSWAYTHGLHHARVNHMLDGESHTPNLQRKVHANFQK
LADLMGDEAFAVLNVFVYLLLAWPLYIINGSGASKRNHEGKRWSKEMLKRPNHFLPTSELFPDKMRLSVASSTAG
VLVVIASLCYWGSIEGSRTVLLQYFLPYLVVNAYLIGFTWMQHTHQDVPHLGEDEVVLGRMNHSHHRSPYPAFID
VLTHRIGSTHVAHHLFSKMPWYHAREATVHIKALLEPKGVYNYDPTPFTRCTTPPDTVTLWRASKAPSSSWTLT
LNLQKQRSSKISSF

CLAIMS:

1. A method of producing a polyunsaturated fatty acid in a host cell comprising the steps of:
   (A) transforming a host cell with a nucleotide sequence comprising: 1) an expression cassette comprising a transcriptional and translational initiation regulatory region, said expression cassette being joined in reading frame 5' to 2) a DNA sequence encoding a desaturase polypeptide which modulates the production of polyunsaturated fatty acids; and
   (B) culturing said transformed host cell under time and conditions sufficient for the expression of said desaturase polypeptide in said host cell, expression of said desaturase polypeptide resulting in production of polyunsaturated fatty acids by said host cell.